The Human ALL-1/MLL/HRX Antigen Is Predominantly Localized in the Nucleus of Resting and Proliferating Peripheral Blood Mononuclear Cells

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ABSTRACT

The ALL-I gene is an important regulator of embryonal and hematopoietic development, and structural variants of the human gene generated by chromosomal translocations and other genetic alterations presumably act as oncogenes in the pathogenesis of acute leukemias and other hematological malignancies. Antisera against two different epitopes of the human ALL-1 protein (anti-ALL-1-N and anti-ALL-1-C) were produced. Both sera revealed indistinguishable patterns of antigen localization in human peripheral blood mononuclear cells (PBMCs). In resting PBMCs, the antigen was distributed in a speckled pattern across the nuclei, with an increased density at the nuclear envelope and the nuclear indentation. In mitotically stimulated PBMCs, the antigen surrounded the condensing mitotic chromosomes but did not colocalize with chromatin or the nuclear scaffold. The antigen is considered a marker for a novel nuclear subcompartment, a perichromosomal area termed the “chromosomal envelope.” In Western blot experiments, the anti-ALL-1-N serum reacted with a polypeptide corresponding to the expected full-length 430-kDa ALL-1 protein. Recombinant proteins representing the AT-hook and zinc binding subdomains of the ALL-1 protein interacted in vivo with a degenerate mixture of double-stranded oligodeoxynucleotides. Thus, the ALL-1 protein probably is a DNA-binding protein with both a sequence-unspecific (AT-hook) and a sequence-specific (zinc binding subdomains) double-stranded DNA binding mode.

INTRODUCTION

Genetic alterations of the human ALL-1/MLL/HRX (ALL-1) gene, located at chromosomal position 11q23, are consistently associated with acute malignancies of hematopoietic cells. Among these are acute lymphoblastic and myeloid leukemias with extremely poor outcome (1—6). The molecular alterations include chromosomal translocations, internal duplications, and specific internal deletions (7—11).

The ALL-1 gene was first mapped to a 92-kb DNA fragment located between the CD36 and Thy-1 loci (12). The complete cDNA has been cloned and sequenced (13, 14), and the exon-intron structure of the ALL-1 gene has been determined recently (15—17). The gene consists of at least 37 exons that encode an open reading frame of 12,018 bp (16, 17). The predicted protein is composed of 4,005 amino acids and shows homology to the Trithorax protein, a regulatory protein involved in Drosophila embryogenesis (18—20). The human ALL-1 protein shares similar length and domains along the protein axis with the Trithorax protein. Knockout experiments of the mouse ALL-1 gene revealed a role of the ALL-1 protein in normal hematopoietic development (21), a homeotically transformed phenotype during embryonal development, and hematopoietic dysfunctions in mice heterozygous for the deletion, whereas a homozygous deletion led to embryonic lethality (22). These findings suggested a similar role in embryogenesis for the murine ALL-1 and Drosophila Trithorax proteins.

Several domains of the human ALL-1 protein have DNA-binding and transcriptional activation activities (23, 24). At the amino-terminal part, AT-hooks and SPKK motifs have been identified (14), representing minor groove DNA binding motifs similar to those present in HMG-Y proteins (25). For the AT-hook motif, a DNA-binding activity to presumably cruciform DNA molecules has already been demonstrated (23). Motifs with homology to cysteine methyltransferase (26) and a predicted bipartite zinc binding motif were located further downstream (13, 14, 27). The predicted bipartite zinc binding domain was categorized as a PHD motif (28), due to homologies with plant homeo domain proteins, with potential DNA-binding and/or protein-protein interacting activities (11).

The aim of the present study was to analyze the intracellular localization of the human ALL-1 protein in normal PBMCs.3 From these experiments, we expected new insights into the functions of the ALL-1 protein in hematopoietic cells. Understanding the functions of the wild-type ALL-1 protein may help to unravel the malignant process in hematopoietic cells that carry one of the known genetic alterations of the ALL-1 gene.

MATERIALS AND METHODS

Cloning of Recombinant Proteins, Isolation and Immunization of Rabbits.

Parts of the ALL-1 cDNA [amino acids 192—503 (AT-hook), amino acids 1428—1513 (PHD-A), amino acids 1428—1463 (PHD-BC), amino acids 1428—1663 (PHD-AB), amino acids 1428—2163 (ALLI-C), and amino acids 3750—4005 (SET)] were cloned into the prokaryotic expression vectors pET15b (Invitrogen, NV Leek, Netherlands) or pGEX-5T (Pharmacia, Freiburg, Germany). The former encodes a HHT, and the latter encodes a HHT and a GST tag, respectively. Recombinant clones were selected and verified by sequence analysis. Fusion proteins were induced by isopropyl-thiogalactoside, and optimal expression was determined by measurement of the induction kinetics. Large-scale preparations were performed for each construct under optimal conditions. Crude extracts were obtained after lysis in 6 M guanidinium hydrochloride and fractionated on nickel-chelate agarose columns (Quiagen, Hilden, Germany). All fractions containing recombinant proteins were sequentially dialyzed against a degenerate mixture of double-stranded oligodeoxynucleotides. Thus, the ALL-1 protein probably is a DNA-binding protein with both a sequence-unspecific (AT-hook) and a sequence-specific (zinc binding subdomains) double-stranded DNA binding mode.

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3 The abbreviations used are: PBMC, peripheral blood mononuclear cell; HHT, hexameric histidine tag; GST, glutathione S-transferase; PHA, phytohemagglutinin; PBT, PBSO.1% Triton X-100; SWPD, South-Western protein dot blot; kDa, kilodalton(s); ds, double-stranded.

2035
The anti-ALL1-N immune serum was affinity-purified by absorption on affinity matrices carrying the recombinant antigen using standard procedures (29).

Cell Isolation and Cell Culture. Human mononuclear cells were isolated from the peripheral blood of healthy donors after informed consent by centrifugation on Ficoll-Hypaque density gradients. Cells were cultured for 72 h in macrocultures at 2 × 10^6 cells/ml in RPMI 1640 containing 10% FCS in the presence of 1% v/v PHA (Life Technologies, Inc., Milan, Italy). PHA-stimulated cells were treated with 10 µg/ml Colcemid (Life Technologies, Inc.) for 2 h. Mitotic preparations were obtained by hypotonic shock (0.07 M KCl and 30 mM glycerol) for 15 min at 37°C.

Immunocytochemistry. Resting and PHA-stimulated PBMCs as well as mitotic preparations were centrifuged for 4 min at 700 rpm in a Shandon II cytopsin centrifuge, and the slides were fixed for 10 min in methanol at −20°C and immediately used for immunocytochemistry. The use of methanol fixation for mitotic preparations, avoiding the classic Carnoy fixation after the hypotonic shock, was preferred to preserve the native structure and antigenicity of cellular components.

Slides with resting or PHA-stimulated cells were rehydrated with PBS and preincubated with phenylhydrazine (10 µM) in PBS to block endogenous peroxidase activity. Slides were rinsed four times in PBS, with the addition of 0.1% Triton X-100 (PBT) in the last wash, and preincubated with normal goat serum (1:10) for 15 min at room temperature. Preparations were incubated with ALL1-C antisera (1:200 in PBT/1% normal goat serum) or affinity-purified ALL1-N antisera (30 µg/ml) for 2 h in a moist chamber. After three wash steps with PBT, the slides were incubated with biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Inc., Burlingame, CA) for 30 min. After three more washes, acetylated avidin-peroxidase was added (1:250 in PBS; Biorad, Milan, Italy). The reaction was developed with diaminobenzidine (1 mg/ml in PBS/0.03% H₂O₂). Negative controls were performed using preimmune sera or irrelevant rabbit IgG as appropriate.

Chromosomal preparations were immunostained with the same procedure, except for using avidin DCS-FITC (1:1000 in PBS; Vector Laboratories, Inc.) after the secondary antibody. Preparations were finally stained with a fluorescence antifade solution containing propidium iodide (0.5 µg/ml) and triethylenediamine (2% w/v; Sigma).

Western Blot Analysis. Mononuclear cells were harvested, washed in PBS, and lysed (20 × 10^6 cells/ml) by adding radioligand immunoprecipitation assay buffer (150 mM NaCl; 20 mM HEPES; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 5 µg/ml leupeptin, pepstatin, chymostatin, and aprotinin; and 1 mM DTT (pH 7.5)). After rotating for 20 min at 4°C, the lysate was centrifuged for 10 min at 14,000 × g, and the supernatant was collected. Protein concentrations were determined using the Bio-Rad Trans-Blot electrophoretic transfer cell (900 mA, 6 h). For immunodetection, the membrane was blocked with 3% BSA in PBS for 16 h at 4°C. To eliminate unspecific binding of radiolabeled ds DNA to immobilized proteins, three wash steps were performed (30 min at 4°C) with a buffer containing 10 mM Tris- HCl (pH 8.0), 50 mM sodium chloride, and 0.5 mM zinc chloride. Subsequently, the membrane was exposed to X-ray film for 2–3 days.

RESULTS

The ALL1 Antigen Is Located Predominantly in the Nucleus of Resting PBMCs. In resting human PBMCs, the anti-ALL1-C and anti-ALL1-N sera generated overlapping patterns of antigen localization (Fig. 1). The antigen was predominantly located in the nuclei, with a speckled distribution across the nuclei and an increased staining intensity near the nuclear envelope. Particularly strong staining was consistently detected near the nuclear indentation (Fig. 1A, inset). Neither irrelevant rabbit IgG nor preimmune sera reacted with these cellular structures (Fig. 1B, inset), whereas an anti-pan-histone antisera generated a pattern of intense nuclear staining with uniform distribution across the nuclei (Fig. 1C). Thus, the ALL1-antigen showed a pattern of nuclear localization that was clearly distinct from the distribution of histones.

In Mitogen-stimulated Human PBMCs, the ALL1 Antigen Is Associated with the Chromosomal Envelope. The localization of the ALL1-antigen in mitogen-stimulated human PBMCs showed remarkable differences from the pattern observed in resting PBMCs. In PHA/Colcemid-treated cells, the anti-ALL1-C serum revealed a distribution of the antigen that was closely associated with chromatin in various stages of mitosis (Fig. 2, A and B). Early in the mitotic cycle, the antigen was still associated predominantly with the nuclear envelope (Fig. 2A, black arrows). In later stages of mitosis, the antigen was associated more with nuclear chromatin and less strongly with the nuclear envelope (Fig. 2, A and B, black triangles). The reactivity of this antisera was completely abolished by incubating the cellular preparations with a molar excess of the same bacterially expressed purified protein fragment that represented the core binding portion of the ALL1-protein and that was used as the immunogen for the generation of this antisera (Fig. 2C).

To analyze the location of the ALL1-antigen with greater precision and to address the question of whether the ALL1-antigen is solely attached to the chromosomal envelope or possibly also attached to chromosomal DNA, preparations of mitotic PBMCs were stained by immunofluorescent techniques (Fig. 3). Using these techniques, the antigen was visualized as a halo surrounding the condensing chromo-
NUCLEAR LOCALIZATION OF THE ALL-1 ANTIGEN

as the chromosomal envelope. This assumption was supported by the loss of reactivity in completely dispersed chromosomes (Fig. 4, A and B, central portions). Disruption of the association between the nuclear matrix and the chromatin led to a complete disappearance of the reactivity, whereas DNA polymerase α remained tightly associated with the chromatin under these conditions (Fig. 4C; Ref. 30). Thus, in mitotically stimulated PBMCs, the ALL-1 antigen did not follow the localization of typical chromatin constituents, such as DNA polym-

Fig. 1. The ALL-1 antigen is located at the nuclear envelope and near the nuclear indentation in resting PBMCs. A, the anti-ALL1-C antiserum produced a predominantly nuclear staining pattern with a speckled distribution of the antigen across the nuclei and an increased local density at the nuclear periphery. Inset, higher magnification showing enrichment of the antigen at the nuclear indentation. B, the staining pattern produced with the anti-ALL1-N antiserum was indistinguishable from that produced with anti-ALL1-C. Inset, a control with irrelevant rabbit IgG produced no specific staining. C, staining of the same cellular preparations with an anti-pan-histone antiserum (Boehringer Mannheim) produced a strong uniform nuclear staining but no detectable local enrichment of the antigen at the nuclear periphery.

Fig. 2. In mitotically stimulated human PBMCs, the ALL-1 antigen follows the localization of condensing chromosomes. A, immunostaining of PHA-stimulated and Colcemid-blocked human PBMCs with the anti-ALL1-C serum. The characteristic staining at the nuclear periphery of resting PBMCs (arrows) progressively disappeared, and the antigen progressively formed clumps at the nuclear centers (arrowhead). B, many metaphases, one prometaphase (arrowhead; A), and a prophase (arrow) were detected. C, incubation of the cellular preparations with a molar excess of the antigen used for generation of the anti-ALL1-C serum effectively abolished the immunological staining of the antigen with the anti-ALL1-C serum. Similar results were obtained with the anti-ALL1-N antiserum.

some during the prophase, metaphase, and telophase stages of mitosis (Fig. 3, A and B). Interphase and early metaphase preparations exhibited reactivity in a perichromosomal zone that surrounded the propidium iodide-counterstained chromosomal DNA (Fig. 3A, white arrow and white triangle). Condensed chromosomes were surrounded by the antigen, suggesting that the antigen may constitute part of a nuclear structure for which thus far no specific term is in common use in the literature. Therefore, it is proposed here to refer to this structure...
NUCLEAR LOCALIZATION OF THE ALL-1 ANTIGEN

Fig. 3. In mitotically stimulated human PBMCs, the ALL-1 antigen colocalizes with the chromosomal envelope, a nuclear subcompartment surrounding mitotic chromosomes. Mitotic chromosomes were prepared by hypotonic shock of PHA-stimulated and Colcemid-blocked human PBMCs. A. preparations from cells in prophase (arrow) and metaphase (arrowhead). B. preparations from cells in telophase. Immunofluorescent staining in A and B with the anti-ALL1-C antiserum, counterstaining of DNA with propidium iodide. The green-yellow fluorescence indicates the presence of the antigen in the chromosomal envelope (definition in the text). C. control with preimmune serum from the same rabbits that were later used to produce the hyperimmune serum. Magnification, ×100.

calculated to be in the 430,000 range (Fig. 5, left four lanes). Irrelevant rabbit IgG did not react with this molecular species. The reactivity of the anti-ALL1-N serum with this 430-kDa molecule was completely abolished when the primary antibody was preincubated for 30 min with a molar excess of the same synthetic peptide that was used as the immunogen for the generation of this antiserum (Fig. 5,

erase α, but was predominantly located in the non-chromatin compartment of the chromosomal envelope.

The ALL-1 Protein Is Present as a 430-kDa Molecule in Human PBMC Extracts. Both the anti-ALL1-N and anti-ALL1-C antisera were used in Western blot experiments with whole-cell extracts from resting and PHA-stimulated human PBMCs. The anti-ALL1-N serum but not the anti-ALL1-C serum reacted with a molecular species of an electrophoretic mobility corresponding to a Mr greater than 360,000,
NUCLEAR LOCALIZATION OF THE ALL-I ANTIGEN

The ALL-I protein has attracted the interest of investigators for two main reasons: (a) structural variants of this protein generated by chromosomal translocations and other alterations of the gene are prime candidates for novel oncoproteins that mediate the pathogenesis of hematological malignancies consistently associated with these chromosomal alterations; and (b) the wild-type ALL-I protein apparently plays an important role in both normal embryogenesis and normal hematopoiesis. Disruption of the murine ALL-I gene by homologous recombination generated mice with homeotic abnormalities and perturbations of hematopoiesis in the heterozygous state and led to embryonic lethality in the homozygous state (22).

The ALL-I protein carries sequence domains that are expected to endow it with DNA-binding capacity and transcriptional transactivator potential. In fact, recombinant fragments of the protein have been shown by other authors to have DNA-binding properties in vitro (23, 24) and, in transfection experiments into mammalian cells, to have transcriptional transactivator potential (23, 24). Therefore, it is reasonable to expect a nuclear location and a function as regulators of gene expression for both the intact ALL-I protein and its presumably oncogenic derivatives. This expectation was reinforced by the observation that the ALL-I protein has a high degree of sequence similarity with the Drosophila Trithorax protein, a confirmed regulator of chromatin structure and of the maintenance of expression of homeotic genes in Drosophila (32). However, although these observations were compelling motivations to study the ALL-I protein and its structural derivatives, thus far, surprisingly little was known about these proteins. The reason for this scarcity of information probably included a low abundance of these proteins in the relevant cells, the difficulty of

Recombinant, Bacterially Expressed Proteins Representing the AT-Hook Protein and Zinc Finger Domains of the ALL-I Protein React in Vitro with ds DNA. Recombinant proteins representing the AT-hook motif, the entire zinc binding domain (PHD-ABC), or three separated overlapping portions of this domain (PHD-A, PHD-AB, and PHD-BC), the ALL-I-C domain, and the SET domain were separately expressed in E. coli and chromatographically purified from bacterial extracts. The HHT-GST protein encoded by the empty pGEX-5T plasmid vector was used as negative control (see “Material and Methods”). All recombinant proteins were applied to nitrocellulose membranes in a grid pattern, and the membrane was then incubated with 120 pmol of a degenerate mixture of radiolabeled ds synthetic oligonucleotides. These oligonucleotides were 50 bp in length, with a core of 18 nucleotides of random sequence flanked by 16 nucleotides of specific sequences on either side to facilitate cloning and detection by PCR (primer binding sites, restriction enzyme cleavage sites; see “Material and Methods”). The mixture contained approximately $7 \times 10^3$ ds DNA molecules with approximately $7 \times 10^3$ different molecules, each present in more than 10$^3$ copies (see “Material and Methods” for details). The recombinant protein containing the AT-hook domain displayed a strong DNA-binding activity, and weaker DNA binding was also observed for the PHD-A, PHD-AB, and PHD-BC proteins, whereas the GST control, ALL-I-C, and the complete PHD-ABC and SET proteins loaded in comparable molar quantities to the filter showed no detectable DNA binding (Fig. 6). Thus, the recombinant AT-hook domain showed detectable DNA binding even to an unselected mixture of ds DNA molecules, suggesting that this pattern was generated by a DNA-binding mode that is not highly sequence-specific.

DISCUSSION

The ALL-I protein has attracted the interest of investigators for two main reasons: (a) structural variants of this protein generated by chromosomal translocations and other alterations of the gene are prime candidates for novel oncoproteins that mediate the pathogenesis of hematological malignancies consistently associated with these chromosomal alterations; and (b) the wild-type ALL-I protein apparently plays an important role in both normal embryogenesis and normal hematopoiesis. Disruption of the murine ALL-I gene by homologous recombination generated mice with homeotic abnormalities and perturbations of hematopoiesis in the heterozygous state and led to embryonic lethality in the homozygous state (22).

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their detection due to their large sizes (above 200 kDa for the derivatives, above 400 kDa for the intact protein), and the nonavailability of antisera of sufficient quality.

The main new results presented in this study are: (a) the generation of two new antisera of high quality and sufficiently high titer to be useful for both immunolocalization and Western blot studies; (b) the observation that the ALL-1 antigen was indeed predominantly located in the nucleus of human leukocytes, as anticipated from the known protein sequence and its DNA-binding domains; and (c) the ability of partial domains of this protein to bind ds DNA even if this was not arranged in potentially cruciform sequences.

Antisera directed against various recombinant cDNA-derived ALL-1 protein fragments had previously been produced by several laboratories including ours. However, in our hands, these sera had failed to detect the intact ALL-1 protein in Western blot experiments with extracts from normal human leukocytes and failed to detect the presumed oncogenic derivatives in extracts from leukemia-derived cell lines with translocations to the ALL-1 gene. The main progress reported here is that a polyclonal rabbit antipeptide antiserum directed against amino acids 446–456 of the ALL-1 protein sequence (13, 14) detected a protein in Western blot experiments with extracts from normal human PBMCs that corresponded in size to the expected 430-kDa intact protein. This presumed full-length protein was at least sufficiently stable in these cells to be detected as the major species in this experiment. It is interesting that the antiserum directed against a purified recombinant protein representing the ALL-1 core (anti-ALL1-C) did not detect this high molecular weight species in the Western blots, although it generated an indistinguishable pattern of antigenic reactivity in immunocytoc hemical experiments as the anti-ALL1-N antiserum (Fig. 1). The reason for this observation is unknown but could simply reflect insufficient affinity of this antiserum.

Other interesting findings reported here for the first time include: (a) the nuclear localization of the wild-type ALL-1 antigen in human PBMCs; and (b) the difference in nuclear staining observed between resting and mitotically stimulated PBMCs, with a localization predominantly near the nuclear envelope in resting cells and a localization predominantly associated with the chromosomal envelope in mitotically stimulated cells.

The molecular basis for this difference in distribution is unknown. It could be explained by a redistribution of the antigen between these two compartments induced by the mitotic stimulation or by the generation of a different set of antigenic products, such as a different set of proteolytic products, with different nuclear sublocalization properties. To distinguish between these and other possible explanations will require the biochemical extraction of the antigen from nuclei in different stages of proliferation and its molecular characterization by protein chemical methods.

The association of the antigen with the nuclear structure termed the chromosomal envelope in mitotically stimulated PBMCs is of particular interest, because this structure had not previously been recognized as a distinct entity and thus had not been given a specific name. If this structure had been a well-known nuclear compartment, then it would have been mandatory to search for an antibody directed against a known marker for this compartment and to demonstrate colocalization of the ALL-1 antigen with this marker. However, no such markers were available; therefore, the ALL-1 antigen as recognized by the anti-ALL1-N and anti-ALL1-C sera becomes itself the prototypical marker for this novel subcompartment. Given the absence of other known components of this structure and the scarcity of functional knowledge about the ALL-1 protein, it would be premature to speculate about the function of this newly recognized nuclear compartment. That this compartment was not identical with chromatin was established by the different staining patterns generated with anti-ALL1 and anti-DNA polymerase α antisera, in which DNA polymerase α is a confirmed marker for chromatin, which is closely associated with the chromosomal scaffold.

Although binding of recombinant fragments of the ALL-1 protein containing the AT-hook domain to DNA had previously been reported by other authors (23), the result presented here represents a step forward in our understanding of this DNA-binding property. These other authors had reported that the AT-hook domain exclusively binds to DNA containing sequences capable of forming cruciform structures. When the same sequences were contained in an arrangement that was capable of forming only linear DNA double strands, no binding was reported (23). Due to the strong binding to a degenerate mixture of ds oligonucleotides observed here (Fig. 6), we concluded that this binding mode probably lacked the high degree of sequence specificity generally observed for proteins, which bind to the major groove. By contrast, AT-hook proteins and others that bind DNA with little sequence specificity generally attach to the minor groove of AT-rich DNA. Thus, the new information presented here is that the AT-hook domain is also capable of binding to ds DNA that does not form cruciform structures, because our sequences were random and not specifically designed to build cruciform structures for which an 18-bp sequence would be too short.

Interestingly, the recombinant proteins representing parts of the so-called zinc binding domain (a domain with sequence homology to zinc finger proteins, as predicted from the cDNA sequence) of ALL-1 also showed evidence of DNA binding in this assay (Fig. 6). However, the binding was much weaker than that observed with AT-hook-containing recombinant fragments. The exact explanation of this finding is unknown. Possible explanations include, among others, both a DNA-binding mode with a higher degree of sequence specificity, as expected from the known binding specificity of other zinc finger proteins, and the presence in this experiment of different molar amounts of the actively DNA-binding species on the filter. To distinguish between these possibilities and other possible explanations will require future isolation of individual DNA-binding target sequences for both the AT-hook and zinc binding domains and analysis of their affinities and specificities. Preliminary data showed that the zinc binding domain of the ALL-1 protein indeed possesses preferential binding properties to specific DNA target sequences. It will also be important in the future to isolate genes containing such binding sites as potential target genes for the regulation of their expression by ALL-1 gene-derived polypeptides.

The current picture does not yet allow us to draw conclusions about the function of the normal ALL-1 protein and its presumably oncogenic derivatives, but the availability of the antisera presented here and the discovery of new DNA-binding abilities will allow future investigators to make progress in providing at least partial answers to these important questions.

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